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(54) **MUTANTS OF BONE MORPHOGENETIC PROTEINS**
MUTANTEN VON KNOCHEN-MORPHOGENESEPROTEINEN
MUTANTS DES PROTEINES MORPHOGENETIQUES OSSEUSES

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- **TRENDS IN GENETICS, vol.8, no.3, 1992, AMSTERDAM NL pages 97 - 102 ROSEN V. ET AL. 'The BMP proteins in bone formation and repair'**
- **GENOMICS, vol.14, 1992 pages 759 - 762 HAHN G. V. ET AL. 'A bone morphogenetic protein subfamily: chromosomal localization of human genes for BMP5, BMP6, and BMP7'**

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Description

[0001] The present invention relates to mutants of bone morphogenetic proteins. These mutants are useful, particularly for use in improved processes for preparation of biologically active dimeric recombinant bone morphogenetic proteins produced in insoluble form from bacterial cell cultures.

BACKGROUND OF THE INVENTION

[0002] A number of proteins referred to in the art as bone morphogenetic proteins (BMPs) have recently been identified which are able to induce bone or cartilage formation when implanted into mammals. For example, Wang et al. in U.S. patent 5,013,649, incorporated herein by reference, describe the DNA sequences encoding bovine and human bone morphogenetic proteins 2A (now bone morphogenetic protein-2) and 2B (now bone morphogenetic protein 4); the corresponding proteins encoded by those DNA sequences, and processes for recombinant production of the BMP-2A (now BMP-2) and BMP-2B (now BMP-4) proteins. Wozney et al., in U.S. 5,106,748, incorporated herein by reference, describe the DNA and amino acid sequences of bovine and human bone morphogenetic protein-5 (BMP-5), along with processes for recombinant production of the BMP-5 proteins. In U.S. 5,187,076, incorporated herein by reference, Wozney et al. disclose DNA sequences, amino acid sequences, and process for recombinant production of human and bovine bone morphogenetic protein-6 (BMP-6). DNA and amino acid sequences encoding bone morphogenetic protein-7 (BMP-7, sometimes referred to as OP-1) and processes for recombinant production of BMP-7 are described in Rosen, et al., U.S. 5,141,905, incorporated herein by reference. DNA sequences encoding BMP-8 are disclosed in PCT publication WO91/18098. DNA sequences encoding BMP-9 are disclosed in PCT publication WO93/00432. These references are herein incorporated by reference. These proteins are expected to have broad medical applicability in treatment of bone and cartilage injuries and disorders in mammals. In order to fulfill the expected medical need for these bone morphogenetic proteins, large quantities of biologically active protein will be needed.

[0003] Recombinant production of the bone morphogenetic proteins is possible both in eukaryotic and prokaryotic cell culture systems. A common occurrence in recombinant production of heterologous proteins in prokaryotic cells, such as bacteria, is the formation of insoluble intracellular precipitates known as inclusion bodies. While the bacteria are generally able to transcribe and to translate DNA sequences encoding heterologous proteins correctly, these prokaryotic cells are unable to fold some heterologous proteins sufficiently correctly to allow for their production in a soluble form. This is particularly true of prokaryotic expression of proteins of eukaryotic origin, such as the bone morphogenetic proteins. Formation of incorrectly folded heterologous proteins has to some extent limited the commercial utility of bacterial fermentation to produce recombinant mammalian proteins. When produced in bacteria, the recombinant bone morphogenetic proteins are often similarly found in inclusion bodies in an aggregated, biologically inactive form.

[0004] Several methods for obtaining correctly folded heterologous proteins from bacterial inclusion bodies are known. These methods generally involve solubilizing the protein from the inclusion bodies, then denaturing the protein completely using a chaotropic agent. When cysteine residues are present in the primary amino acid sequence of the protein, it is often necessary to accomplish the refolding in an environment which allows correct formation of disulfide bonds (a redox system). General methods of refolding are disclosed in Kohno, Meth. Enzym., 185:187-195 (1990).

[0005] EP 0433225 describes a method for refolding transforming growth factor β (TGF- β)-like proteins which employs, in addition to a chaotropic agent and a redox system, a solubilizing agent in the form of a detergent. EP 0433225 predicts that the methods disclosed therein are generally applicable for refolding "TGF- β -like proteins", based on the degree of homology between members of the TGF- β family. However, the present inventors have found that the methods disclosed in EP 0433225 produce undesirably low yields of correctly folded, biologically active dimeric protein when applied to bacterially produced BMP-4, BMP-5, BMP-6, or BMP-7 for unknown reasons.

SUMMARY OF THE INVENTION

[0006] It has been found, unexpectedly, that although some bone morphogenetic proteins do not yield correctly folded, biologically active dimeric protein when produced bacterially, such as BMP-4, BMP-5, BMP-6, BMP-7, or BMP-8 certain mutant forms of these proteins are able to yield such proteins. It has further been found, also unexpectedly, that certain mutant forms of bone morphogenetic proteins are also able to yield correctly folded, biologically active heterodimers, such as heterodimers of BMP-2/5 and BMP-2/6, in good quantity, whereas the native forms of these proteins produce undesirably low yields of correctly folded, biologically active heterodimers, yields which are improved by the methods of this invention.

[0007] Accordingly, in one embodiment, the invention comprises mutant forms of BMP-4 which are useful in bacterial production processes for yielding correctly folded, biologically active forms of BMP-4.

[0008] In another embodiment, the invention comprises mutant forms of BMP-5, BMP-6, BMP-7 and BMP-8 which

are useful in bacterial production processes for yielding correctly folded, biologically active forms of heterodimers of BMP-2/5, BMP-2/6, BMP-2/7 and BMP-2/8.

[0009] In a further embodiment, the invention comprises DNA molecules comprising DNA sequences encoding the above mutant forms of bone morphogenetic proteins.

5 [0010] The present invention further comprises a method for obtaining other mutants of bone morphogenetic proteins with improved refolding properties, and the mutant proteins thereby obtained.

BRIEF DESCRIPTION OF THE SEQUENCES

10 [0011]

SEQ ID NO:1 is the nucleotide sequence encoding BMP-2.

SEQ ID NO:2 is the amino acid sequence for BMP-2.

SEQ ID NO:3 is the nucleotide sequence encoding BMP-4.

15 SEQ ID NO:4 is the amino acid sequence for BMP-4.

SEQ ID NO:5 is the nucleotide sequence encoding BMP-5.

SEQ ID NO:6 is the amino acid sequence for BMP-5.

SEQ ID NO:7 is the nucleotide sequence encoding BMP-6.

SEQ ID NO:8 is the amino acid sequence for BMP-6.

20 SEQ ID NO:9 is the nucleotide sequence encoding BMP-7.

SEQ ID NO:10 is the amino acid sequence for BMP-7.

SEQ ID NO:11 is the nucleotide sequence encoding BMP-8.

SEQ ID NO:12 is the amino acid sequence for BMP-8.

25 DESCRIPTION OF THE FIGURE

[0012] Figure 1 is a comparison of sequences of BMP-2, 4, 5, 6, 7 and 8.

DETAILED DESCRIPTION OF THE INVENTION

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[0013] In accordance with the present invention, mutant forms of recombinant bone morphogenetic protein-4 (BMP-4) (SEQ ID NO:3 and 4); BMP-5 (SEQ ID NO:5 and 6); BMP-6 (SEQ ID NO:7 and 8); BMP-7 (SEQ ID NO:9 and 10); and BMP-8 (SEQ ID NO:11 and 12) may be used to produce large quantities of BMP homodimers or heterodimers from bacteria and refolded into biologically active dimeric molecules.

35 [0014] The DNA molecules of the present invention include DNA molecules comprising a nucleotide sequence encoding BMP-4, except that the nucleotide triplet encoding glutamic acid at residue 107 (i.e., nucleotides 319 to 321 of SEQ ID NO:3) is replaced (for example, by mutation or synthetically) by a nucleotide triplet that encodes an aspartic acid at residue 107.

40 [0015] Another embodiment of the present invention comprises DNA molecules comprising a nucleotide sequence encoding BMP-5, BMP-6, or BMP-7, except that the nucleotide triplet encoding alanine at residue 56 of BMP-5 or BMP-6 (i.e., nucleotides 166 to 168) of SEQ ID NO:5 or 7), or residue 63 of BMP-7 (i.e., nucleotides 187 to 189 of SEQ ID NO:9), is replaced (for example, by mutation or synthetically) by a nucleotide triplet that encodes a histidine.

45 [0016] Another embodiment of the present invention comprises DNA molecules comprising a nucleotide sequence encoding BMP-8, except that the nucleotide triplet encoding serine at residue 63 of BMP-8 (i.e., nucleotides 187 to 189 of SEQ ID NO: 11), is replaced (for example, by mutation or synthetically) by a nucleotide triplet that encodes a histidine.

[0017] The present invention further comprises purified compositions of protein comprising the amino acid sequence of BMP-4, except that the amino acid glutamic acid at residue 107 is replaced by an aspartic acid. This modified BMP-4 protein may be referred to by the nomenclature BMP-4(Δ 107Asp).

50 [0018] In another embodiment, the present invention comprises purified compositions of protein comprising the amino acid sequences of BMP-5, BMP-6 or BMP-7, except that the amino acid alanine at residue 56 of BMP-5 or BMP-6, or residue 63 of BMP-7, is replaced by a histidine. The modified BMP-5 protein may be referred to, for example, by the nomenclature BMP-5(Δ 56His). In another embodiment, the present invention comprises purified compositions of protein comprising the amino acid sequences of BMP-8, except that the amino acid serine at residue 63 of BMP-8, is replaced by a histidine. The modified BMP-8 protein may be referred to, for example, by the nomenclature BMP-8(Δ 63His).

55 [0019] As used herein, the term "correlative" means the following. It is known that BMP-2 comprises a dimer of polypeptide chains, each of which may be 114 amino acids in length. Similarly, BMP-4 comprises a dimer of polypeptide

chains, each of which may be 116 amino acids in length. BMP-5 and BMP-6 each comprise dimers of polypeptide chains, each of which may be 132 amino acids in length. BMP-7 comprises a dimer of polypeptide chains, each of which may be 139 amino acids in length. BMP-8 comprises a dimer of polypeptide chains, each of which may be 139 amino acids in length. It is further known that the amino acids of BMP-2 from the leucine at residue 19 (correlative to residue 21 of BMP-4) through arginine at residue 114 is highly homologous to BMP-4 from leucine at residue 21 to arginine at residue 116). Similarly, it is known that the amino acids of BMP-2 from leucine 19 of BMP-2 through arginine 114 of BMP-2 are highly homologous to amino acids leucine 36 through histidine 132 of BMP-5 and BMP-6 and amino acids leucine 43 through histidine 139 of BMP-7, and to leucine 43 to histidine 139 of BMP-8. Thus, the leucine at residue 19 of BMP-2 is said to be correlative to residue 21 of BMP-4, and to residues 36 of BMP-5 and BMP-6, and to residue 43 of BMP-7, and to residue 43 of BMP-8. Similarly, the aspartic acid at residue 105 of BMP-2 is said to be correlative to the glutamic acid at residue 107 of BMP-4, and the histidine at residue 39 of BMP-2 is said to be correlative to the alanine at residues 56 of BMP-5 and BMP-6 and the alanine at residue 63 of BMP-7, and the serine at residue 63 of BMP-8. Alternatively, the 112 amino acid sequence of TGF- β may also be used as a reference point for defining correlative amino acids.

[0020] From an examination of Figure 1, it can be seen that BMP-2 and BMP-4 are highly homologous, beginning at the first cysteine (residue 14 of BMP-2; correlative residue 16 of BMP-4). There are only eight correlative residues which are different. These are, respectively, at residues 15, 39, 46, 73, 95, 96 and 105 of BMP-2. Yet, Applicants have found that the methods disclosed in EP 0433225, which are effective for refolding BMP-2 in acceptable quantities, produce undesirably low yields of correctly folded, biologically active dimeric protein when applied to bacterially produced BMP-4. Applicants constructed molecules in which the first four (N-terminal) of these residues resembled the BMP-2 residue, while the last four (C-terminal) of these residues resembled the correlative BMP-4 residue (called "BMP-2/BMP-4"). Applicants also constructed molecules in which the N-terminal four of these residues resembled BMP-4, while the C-terminal four of these residues resembled the correlative BMP-4 residue (called "BMP-4/BMP-2"). As described in Example 2, Applicants found that while BMP-4/BMP-2 refolded in good quantity, BMP-2/BMP-4 did not.

[0021] The present invention includes DNA molecules comprising a DNA sequence encoding BMP-4, wherein at least the nucleotide sequence encoding the amino acid glutamic acid at residue 107 is replaced by the correlative nucleotide sequence of BMP-2 encoding aspartic acid. In addition, it is contemplated that other nucleotide sequences of BMP-4 may be replaced by the correlative nucleotide sequence of BMP-2, so long as the glutamic acid residue at 107 is replaced by the correlative aspartic acid residue of BMP-2. Such a DNA molecule may be chimeric, that is, portions of BMP-2 coding sequence and BMP-4 coding sequence may be ligated together through methods readily known to those skilled in the art. Alternatively, this DNA molecule may be constructed synthetically or through mutations, such as by chemical means. The DNA molecule, once formed can be dimerized through methods known in the art, either with itself (homodimer) or with a different member of the BMP family (heterodimer).

[0022] The present invention further includes DNA molecules comprising a DNA sequence encoding BMP-5, BMP-6, BMP-7, or BMP-8 wherein the nucleotide sequence encoding the amino acid alanine at residue 56 of BMP-5 or BMP-6, or residue 63 of BMP-7 or BMP-8, is replaced by the correlative nucleotide sequence of BMP-2. In addition, it is contemplated that other nucleotide sequences of BMP-5, BMP-6, BMP-7 or BMP-8 may be replaced by the correlative nucleotide sequence of BMP-2, so long as the alanine residue at 56 (63 of BMP-7), or serine residue at 63 of BMP-8, is replaced by the correlative histidine residue of BMP-2. Such a DNA molecule may be chimeric, that is, portions of BMP-2 coding sequence and BMP-5, BMP-6, BMP-7 or BMP-8 coding sequence may be ligated together through methods readily known to those skilled in the art. Alternatively, this DNA molecule may be constructed synthetically or through mutations, such as by chemical means. The DNA molecule, once formed can be dimerized through methods known in the art.

[0023] The present invention further comprises methods of obtaining other mutants of bone morphogenetic proteins (BMP) with improved refolding properties, and the mutant proteins thereby obtained. The method comprises first comparing the amino acid sequence of a BMP which is found to refold well (*BMP**) using the refolding methods described herein, with the amino acid sequence of a BMP which does not refold well using such methods (*BMP*), and the differences at correlative amino acid positions are determined. Next, the amino acid sequence of *BMP* is altered so that one or more amino acids different from those of correlative amino acids of *BMP** are replaced by the correlative amino acids of *BMP**. For example, such modified amino acids could be formed by creating one or more nucleotide mutations or substitutions in the DNA sequence encoding the amino acid sequence for *BMP* so that the DNA sequence will express a modified *BMP* protein. The modified *BMP* protein is then tested for its ability to refold. This method may be repeated for each amino acid position at which the sequence of *BMP** and *BMP* differ in order to identify those amino acid residues that are critical to the differences in refolding. Further, multiple changes to the amino acid sequence of *BMP* may be made to replace amino acid residues with the correlative amino acid from *BMP** in order to further improve the refolding of the modified *BMP* protein. The modified *BMP* proteins, and the DNA sequence encoding them, are also within the present invention.

[0024] Methods of mutagenesis of proteins and nucleic acids are known, for example see Sambrook et al., Molecular

Cloning: A Laboratory Manual, 2d ed. (Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press)(1990). It is further known that there may exist more than one nucleotide triplet that encodes a given amino acid residue. For example, a histidine residue may be encoded by either CAT or CAC, and an aspartic acid residue may be encoded by either GAT or GAC. See Lehninger, Biochemistry, (Worth Publishers, N.Y., N.Y.)

5 [0025] Any bacterial species may be used to generate recombinant BMP for refolding in the method of the invention. Preferably, *Bacillus subtilis* is used to produce inclusion bodies containing BMP. More preferably, *Pseudomonas* is used to produce inclusion bodies containing BMP for refolding in the method of the invention. Most preferably, *Escherichia coli* is used to produce inclusion bodies containing BMP for refolding in the method of the invention. Any strain of *E. coli* may be used to produce BMP for refolding in the method of the invention, so long as that strain is capable of expression of heterologous proteins. One preferred strain, *E. coli* strain G1724 (A.T.C.C. accession number 55151) may be used to produce BMP for refolding in the method of the invention.

10 [0026] The mutant forms of BMP of the present invention may be produced in bacteria using known methods. It may be necessary to modify the N-terminal sequences of the mutant forms of BMP in order to optimize bacterial expression. For example, because cleavage of the bond between formyl-methionine and glutamine is inefficient in *E. coli*, the N-terminus of the native mature BMP-2 protein (Met-gln-ala-lys) is modified by deletion of the glutamine residue to yield an N-terminus more suitable for BMP-2 production in *E. coli* (Met-ala-lys-his). Other bacterial species may require analogous modifications to optimize the yield of the mutant BMP obtained therefrom. Such modifications are well within the level of ordinary skill in the art.

15 [0027] The modified or unmodified nucleotide sequence of SEQ ID NO:3 which encodes BMP-4; SEQ ID NO:5, which encodes BMP-5; SEQ ID NO:7, which encodes BMP-6; SEQ ID NO:9, which encodes BMP-7, or SEQ ID NO: 11, which encodes BMP-8, may be inserted into a plasmid suitable for transformation and expression of those heterologous proteins in bacteria. Any bacterial expression plasmid may be used, so long as it is capable of directing the expression of a heterologous protein such as BMP in the bacteria chosen. Acceptable species of bacteria include *B. subtilis*, species of *Pseudomonas*, and *E. coli*. Suitable expression plasmids for each of these species are known in the art. For production of BMP in bacteria, a suitable vector is described in Taniguchi et al., *PNAS:USA*, 77:5230-5233 (1980).

20 [0028] The bacterial expression plasmid may be transformed into a competent bacterial cell using known methods. Transformants are selected for growth on medium containing an appropriate drug when drug resistance is used as the selective pressure, or for growth on medium which is deficient in an appropriate nutrient when auxotrophy is used as the selective pressure. Expression of the heterologous protein may be optimized using known methods. The BMP thus obtained will be present in insoluble, refractile inclusion bodies which may be found in pellets of disrupted and centrifuged cells.

25 [0029] The inclusion bodies thus obtained are then solubilized using a denaturant or by acidification with acetic acid or trifluoroacetic acid. If solubilized using a denaturant, a reducing agent such as β -mercaptoethanol or dithiothreitol is added with the denaturant. If the protein is solubilized by acidification, it must be reduced prior to acidification. The solubilized heterologous protein may be further purified using known chromatographic methods such as size exclusion chromatography, or exchange chromatography, or reverse phase high performance liquid chromatography.

30 [0030] The solution containing the BMP is then reduced in volume or vacuum desiccated to remove chromatography buffer, and redissolved in medium [suitable media include 50 mM Tris, 1.0 M NaCl, 2% 3-(3-chloramidopropyl)dimethylammonio-1-propane-sulfate (CHAPS), 5 mM EDTA, 2 mM glutathione (reduced) 1 mM glutathione (oxidized); at pH of approximately 8.5; other media which may be suitable for redissolution include alternative refolding buffers described elsewhere in the specification (e.g., guanidine, urea, arginine)] to yield a concentration of 1 to 100 μ g/ml protein. Higher concentrations of protein may be refolded in accordance with the invention, for example up to about 1 mg/ml, but precipitates or aggregates are present above protein concentrations of 100 μ g/ml and the yield of active BMP homodimer or heterodimer may be decreased accordingly.

35 [0031] For production of heterodimers, the above procedure is performed utilizing equal amounts of two plasmids, each containing a coding sequence for a distinct BMP (e.g., pALBP2, encoding BMP-2 and pALBPX encoding BMP-X, where X is 5, 6, 7 or 8). The plasmids are cultured separately, and the resulting inclusion bodies are solubilized and refolded in accordance with the methods described herein. The refolded protein monomers are mixed together in equivalent ratios and treated as described in the paragraph above. For heterodimers, the media uses CHAPS as the refolding buffer. The resulting dimeric proteins are observed to include homodimers of BMP-2, as well as heterodimers of BMP-2/X. These species may be separated out from each other through procedures known in the art. The production of heterodimers of BMP is more thoroughly described in WO93/09229, the disclosure of which is hereby incorporated by reference.

40 [0032] In order to refold the proteins, the following conditions and media may be used: 50 mM Tris, 1.0 M NaCl, 2% 3-(3-chloramido-propyl)dimethylammonio-1-propane-sulfate (CHAPS), 5 mM EDTA, 2 mM glutathione (reduced) 1 mM glutathione (oxidized); at pH of approximately 8.5. With minor modifications, other detergents, including non-ionic, e.g. digitonin, or zwitterionic detergents, such as 3-(3-chloramidopropyl)dimethylammonio-1-propane-sulfonate

(CHAPSO), or N-octyl glucoside, may be used in the present invention. One skilled in the art will recognize that the above conditions and media may be varied, for example, as described below. Such variations and modifications are within the present invention.

[0033] Because BMPs are disulfide bonded dimers in their active state, it is useful to include a redox system which allows formation of thiol/disulfide bonds in the method of the invention. Several such redox systems are known. For example the oxidized and reduced forms of glutathione, dithiothreitol, β -mercaptoethanol, β -mercaptomethanol, cystine and cystamine may be used as redox systems at ratios of reductant to oxidant of about 1:10 to about 2:1. When the glutathione redox system is used, the ratio of reduced glutathione to oxidized glutathione is preferably 0.5 to 5; more preferably 1 to 1; and most preferably 2 to 1 of reduced form to oxidized form.

[0034] With additional modifications, other refolding agents, such as urea, guanidine, arginine and other means of refolding, may be useful in order to produce correctly refolded proteins with the mutants of the present invention. Chaotropic agents are generally used at concentrations in the range of 1 to 9M. When urea is the refolding agent, it is preferably present at concentrations in the range of about 0.1M to about 3M, more preferably about 0.5M to 2.5M, or about 1.0M to about 2.0M.

[0035] When guanidine hydrochloride is used as the refolding agent, it is preferably initially added at high concentrations, for example, 7-8M, and then the concentration of guanidine is reduced to induce refolding. The reduction of guanidine concentration may occur instantaneously, as by dilution, or gradually, as by dialysis. Preferably the guanidine concentration is reduced to a final concentration of less than about 1.5M, or more preferably less than about 1M. When the guanidine concentration is reduced gradually, the guanidine may be completely removed from the refolded protein.

Dilution of a guanidine is preferable over dialysis.

[0036] When arginine is used as the refolding agent, it is preferably present at concentrations of about 0.4M to about 1.5M, more preferably, about 0.6M to about 1.25M, or about 0.6M to about 1.0M.

[0037] In addition to the refolding agent, the method of the invention may employ a salt moiety. When detergents, such as CHAPS, are used, the salt moiety is preferably NaCl, preferably at a concentration of about 0.5M to about 2.0M, preferably about 1.0M. When urea is the refolding agent, the salt moiety is preferably sodium chloride, preferably at a concentration of about 0.25M to about 2M. More preferably, the sodium chloride is present at a concentration in the range of about 0.5M to about 1.5M when urea is the refolding agent. Most preferably, when urea is the refolding agent, sodium chloride is present at a concentration in the range of about 0.75M to about 1.25M. When guanidine is used as the refolding agent, the sodium chloride concentration must be increased as the concentration of guanidine increases. For example, for refolding in 0.2M guanidine, the range of NaCl concentration which is optimal is 0.25 to 0.5M, while for refolding in 1.0M guanidine, 1.0 to 2.0M NaCl is necessary for optimal refolding.

[0038] The pH of the refolding reaction of the present invention when urea is the refolding agent is preferably from about 7.5 to about 11; more preferably from about 8.5 to about 10.5. When detergents such as CHAPS, are used as the refolding agent, the preferred pH is about 8.5. When guanidine is used as the refolding agent, the pH is preferably from about 7.5 to about 9.5; more preferably about 8.5; and most preferably about 9.5. When arginine is used as the refolding agent, the pH is preferably from about 8 to about 10; more preferably from about 8.5 to about 10; and most preferably from about 9.5 to about 10.

[0039] Preferably, the refolding reaction of the invention is performed at a temperature range from about 4°C to about 23°C. More preferably, the refolding reaction is performed at 4°C. The refolding reactions of the present invention are allowed to proceed to completion, preferably about 16 hours.

[0040] The extent of refolding of bone morphogenetic proteins obtained is monitored by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) under non-reduced and reduced conditions. The BMP-4 homodimer will appear as a band of about 30 kD under non-reduced conditions on a 16 percent SDS-polyacrylamide gel; and the BMP-4 monomer appears as a band of about 13 kD under reduced conditions. The BMP-2/5 heterodimer will appear as a band of about 35 kD under non-reduced conditions on a 16 percent SDS-polyacrylamide gel; the BMP-2 monomer appears as a band of about 13 kD under reduced conditions; and the BMP-5 monomer appears as a band of about 15 kD under reduced conditions. The BMP-2/6 heterodimer will appear as a band of about 35 kD under non-reduced conditions on a 16 percent SDS-polyacrylamide gel; the BMP-2 monomer appears as a band of about 13 kD under reduced conditions; and the BMP-6 monomer appears as a band of about 15 kD under reduced conditions. The BMP-2/7 heterodimer will appear as a band of about 35 kD under non-reduced conditions on a 16 percent SDS-polyacrylamide gel; the BMP-2 monomer appears as a band of about 13 kD under reduced conditions; and the BMP-7 monomer appears as a band of about 15 kD under reduced conditions.

[0041] The *in vitro* biological activity of the refolded bone morphogenetic proteins is monitored by the W-20 assay as set forth in Example 9. Use of the W-20-17 bone marrow stromal cells as an indicator cell line is based upon the conversion of these cells to osteoblast-like cells after treatment with BMP [R. S. Thies et al., *Journal of Bone and Mineral Research* 5(2):305 (1990); and R. S. Thies et al., *Endocrinology* 130:1318-1324 (1992)]. W-20-17 cells are a clonal bone marrow stromal cell line derived from adult mice by researchers in the laboratory of Dr. D. Nathan, Children's Hospital, Boston, MA. Treatment of W-20-17 cells with BMP results in (1) increased alkaline phosphatase production,

(2) induction of parathyroid hormone stimulated cAMP, and (3) induction of osteocalcin synthesis by the cells. While (1) and (2) represent characteristics associated with the osteoblast phenotype, the ability to synthesize osteocalcin is a phenotypic property only displayed by mature osteoblasts. Furthermore, to date the conversion of W-20-17 stromal cells to osteoblast-like cells has been observed only upon treatment with bone morphogenetic proteins. The *in vivo* biological activity of the refolded bone morphogenetic proteins is monitored by a modified version of the rat bone formation assay described in Sampath and Reddi, Proc. Natl. Acad. Sci. USA, 80:6591-6595 (1983) herein called the Rosen-modified Sampath-Reddi assay, as set forth in Example 10.

Example 1

Refolding of BMP-4 using CHAPS system

[0042] 1.0 g of cells stored at -80°C are measured. Solution (3.4 ml 100mM TRIS, 10mM EDTA, pH 8.5) is added. The solution is vortexed until cells are well suspended. 40 µl 100 mM PMSF in isopropanol is added. The cells are lysed at 1000 psi in a French pressure cell. The inclusion bodies are centrifuged at 4°C for 20 minutes in an Eppendorf microfuge to form pellets. The supernatants are decanted. To one pellet (out of 4 total) 1.0 ml degassed 8.0M guanidine hydrochloride, 0.5M TRIS, 5mM EDTA, pH 8.5, containing 250mM DTT is added. The pellet is dissolved and argon is blown over the liquid for 30 seconds. Next the solution is incubated at 37°C for one hour. Insoluble material is pelleted for 2-3 minutes in an Eppendorf microfuge at 23°C. 0.5-1.0 ml of supernatant is injected onto a Supelco 2 cm guard cartridge (LC-304), and eluted with an acetonitrile gradient in 0.1 % TFA from 1-70% over 35 minutes. BMP-4 elutes between 30 and 32 minutes. Fractions are pooled and the protein concentration determined by A280 versus 0.1 % TFA, using the theoretical extinction coefficient based upon the amino acid content.

[0043] A sufficient volume of the BMP-4 pool is lyophilized to give 10 µg of protein. 5 µl of glass distilled water is added to redissolve the residue, then 100 µl of refold mix (TRIS, salt, CHAPS, etc.) is added. The solution is gently mixed and stored at 23 °C for 1-4 days. Dimer formation is assessed by running an aliquot on a Novex 16% tricine gel at 125 volts for 2.5 hours, followed by Coomassie Blue staining and destaining.

Example 2

Refolding of other BMP dimers

[0044] From an examination of Figure 1, it can be seen that BMP-2 and BMP-4 are highly homologous, beginning at the first cysteine (residue 14 of BMP-2; correlative residue 16 of BMP-4). There are only eight correlative residues which are different. These are, respectively, at residues 15, 39, 46, 73, 95, 96 and 105 of BMP-2. Yet, Applicants have found that BMP-4 that the methods disclosed in EP 0433225, which are effective for refolding BMP-2 in acceptable quantities, produce undesirably low yields of correctly folded, biologically active dimeric protein when applied to bacterially produced BMP-4. Applicants constructed molecules in which the first four (N-terminal) of these residues resembled the BMP-2 residue, while the last four (C-terminal) of these residues resembled the correlative BMP-4 residue (called "BMP-2/BMP-4"). Applicants also constructed molecules in which the N-terminal four of these residues resembled BMP-4, while the C-terminal four of these residues resembled the correlative BMP-4 residue (called "BMP-4/BMP-2"). These molecules were worked up as described for wild-type BMP-4 above. Gels were run with the appropriate control proteins (e.g., the BMP-4 mutants next to wild-type BMP-4; BMP-2 and wild-type BMP-5 mixed together as a control for the BMP-2 and BMP-5(Δ56His).

[0045] Wild-type BMP-4 did not refold well. While BMP-4/BMP-2 refolded in good yield; however, BMP-2/BMP-4 does not. BMP-4(Δ107Asp) homodimer refolds in good quantity relative to wild-type BMP-4.

[0046] BMP-2/BMP-5 heterodimer does not refold well. BMP2/BMP5(Δ39His) heterodimer refolds in good quantity relative to BMP-2/BMP-5.

[0047] BMP-2/BMP-6 heterodimer does not refold well. BMP2/BMP6(Δ39His) heterodimer refolds in good quantity relative to BMP-2/BMP-6.

Example 3

Expression of BMP in *E. coli*

[0048] An expression plasmid pALBP2-782 containing the following principal features was constructed for production of BMP-2 in *E. coli*. Nucleotides 1-2060 contain DNA sequences originating from the plasmid pUC-18 [Norrand et al., Gene 26:101-106 (1983)] including sequences containing the gene for β-lactamase which confers resistance to the antibiotic ampicillin in host *E. coli* strains, and a colEI-derived origin of replication. Nucleotides 2061-2221 contain

DNA 5 sequences for the major leftward promoter (pL) of bacteriophage λ [Sanger et al., J. Mol. Biol. 162:729-773 (1982)], including three operator sequences O_L1 , O_L2 and O_L3 . The operators are the binding sites for λ cl repressor protein, intracellular levels of which control the amount of transcription initiation from pL. Nucleotides 2222-2723 contain a strong ribosome binding sequence included on a sequence derived from nucleotides 35566 to 35472 and 38137 to 38361 from bacteriophage lambda as described in Sanger et al., J. Mol. Biol. 162:729-773 (1982). Nucleotides 2724-3133 contain a DNA sequence encoding mature BMP-2 protein with an additional 62 nucleotides of 3'-untranslated sequence. Nucleotides 3134-3149 provide a "Linker" DNA sequence containing restriction endonuclease sites. Nucleotides 3150-3218 provide a transcription termination sequence based on that of the *E. coli* *asp* A gene [Takagi et al., Nucl. Acids Res. 13:2063-2074 (1985)]. Nucleotides 3219-3623 are DNA sequences derived from pUC-18.

[0049] Using restriction endonucleases and procedures known in the art, one can readily replace the coding sequence for BMP-2 contained in pALBP2-781 with the coding sequence for another BMP desired to be produced in *E. coli*. With this substitution in the pALBP2-781 plasmid, the following examples may be used to express and refold any of the BMPs of the present invention. Plasmid pALBP2-781 was transformed into the *E. coli* host strain GI724 (F, *lacI*^q, *lacp*^{LB}, *ampC*::*lacI*⁺) by the procedure of Dagert and Ehrlich, Gene 6:23 (1979). GI724 (ATCC accession No. 55151) contains a copy of the wild-type λ cl repressor gene stably integrated into the chromosome at the *ampC* locus, where it has been placed under the transcriptional control of *Salmonella typhimurium* *trp* promoter/operator sequences. In GI724, λ Cl protein is made only during growth in tryptophan-free media, such as minimal media or a minimal medium supplemented with casamino acids such as IMC, described above. Addition of tryptophan to a culture of GI724 will repress the *trp* promoter and turn off synthesis of λ cl, gradually causing the induction of transcription from pL promoters if they are present in the cell.

[0050] Transformants were selected on 1.5 % w/v agar plates containing IMC medium, which is composed of M9 medium [Miller, "Experiments in Molecular Genetics," Cold Spring Harbor Laboratory, New York (1972)] supplemented with 1mM $MgSO_4$, 0.5% w/v glucose, 0.2% w/v casamino acids and 100 μ g/ml ampicillin and GI724 transformed with pALBP2-781 was grown at 370 C to an A_{550} of 0.5 in IMC medium containing 100 μ g/ml ampicillin. Tryptophan was then added to a final concentration of 100 μ g/ml and the culture incubated for a further 4 hours on ampicillin-containing medium. During this time BMP protein accumulates to approximately 10% of the total cell protein, all in the "inclusion body" fraction.

[0051] Nine grams of frozen cell pellets obtained from the *E. coli* transformants as described above were thawed in 30 ml of TE8.3(100:10) buffer (100 mM Tris-HCl pH 8.3, 10 mM Na_2EDTA , 1 mM phenylmethylsulfonyl fluoride (PMSF)). Cells were lysed by three passes through a Microfluidizer™ [model #MCF 100 T]. The lysate was diluted to approximately 120 ml with TE8.3 100:10 buffer. A pellet of inclusion body material was obtained by centrifugation at 15,000 x g. The supernatant was decanted, and the inclusion body material was suspended in 50 ml TE8.3(100:10) which also contained 1% Triton-X100. The resuspended inclusion bodies were centrifuged for 10 minutes at 15,000 x g, and the supernatant was decanted. The pellet was suspended in TE8.3(20:1) buffer (20 mM Tris-HCl pH 8.3, 1 mM Na_2EDTA , 1 mM PMSF) which also contained 1% dithiothreitol (DTT). After the suspension was homogenized in a Wheaton glass homogenizer, it was acidified to pH 2.5 with glacial acetic acid and then centrifuged 25 minutes at 15,000 x g. The supernatant from this centrifugation was collected and chromatographed over a Sepharose S-100™ size exclusion column (83 cm x 2.6 cm; \approx 440 ml bed) in 20 ml increments. The Sepharose S-100™ column was run with a mobile phase of 1% acetic acid at a flow rate of 1.4 ml/min. Fractions corresponding to BMP-2 monomer were detected by absorbance at 280 nm, and using a computer calculated extinction coefficient of 18200M⁻¹cm⁻¹ and molecular weight (12777 daltons). This size exclusion column pooled material was used as starting material for refolding reactions.

[0052] Alternatively, cells were lysed as above, but the initial inclusion body material pellet was dissolved in 8 M guanidine-HCl, TE8.5(100:10) buffer (100 mM Tris-HCl pH 8.5, 10 mM Na_2EDTA which contained 100 mM DTT, and incubated at 37°C for 1 hour. This material was centrifuged at 12,000 x g for 15 minutes at room temperature. The supernatant was injected onto C4 analytical RP-HPLC (reversed phase-high performance liquid chromatography) column (Vydac 214TP54) equilibrated to 1 % B buffer (A buffer - 0.1 % trifluoroacetic acid, B buffer = 95% acetonitrile, 0.1% trifluoroacetic acid (TFA)), with a flow rate of 1 ml/min. After 5 minutes, a linear gradient from 1% to 70% B buffer (diluted into A buffer) was run over 35 minutes, during which time the protein elutes. Protein was monitored by absorbance at 280nm. Peak BMP-2 fractions (eluting between 25 and 35 minutes) were pooled. The concentration was determined by absorbance at 280nm, and using the computer calculated extinction coefficient and molecular weight as indicated above. This RP-HPLC C4 Column pooled material was also used as starting material for refolding reactions.

Example 4Refolding of *E. coli* Produced BMP-2 in Urea/NaCl

- 5 [0053] BMP-2 protein in 1 % acetic acid or in reverse phase buffer containing 0.1 % TFA, 30-40% acetonitrile was dried or reduced in volume using a speed vacuum, redissolved with a few microliters of 0.01 % TFA, and allowed to dissolve completely for 5 to 10 minutes. A buffer containing 7M to 8M urea, 100 mM 2-(N-cyclohexylamino)-ethanesulfonic acid [CHES] pH 9.5, 5 mM EDTA was added to the BMP-2 in TFA and allowed to incubate for 20 minutes at room temperature (RT, approximately 23°C) before dilution. The protein concentrations used were such that the final BMP-2 concentration in the diluted state was 10 to 100 µg/ml. The final conditions of the folding buffer contained 100 mM CHES, 5 mM EDTA, and the desired concentration of salt for the urea concentration used. Several ranges of urea, NaCl, pH, and redox conditions were tested to optimize BMP-2 refolding conditions.
- 10 [0054] Refolding of the *E. coli* produced BMP-2 in urea/NaCl was analyzed under reducing and non-reducing conditions using 16% Tricine-sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE).
- 15 [0055] Refolding was scored as positive when the BMP-2 appeared as a dimer of the appropriate molecular weight under non-reducing conditions and as a monomer of appropriate molecular weight under reducing conditions. Yield of refolded BMP-2 was determined by scanning bands on loomassie blue or silver stained gels. Biological activity of the refolded BMP-2 dimer was tested using the assays of Examples 9 and 10 below.
- 20 [0056] Refolding of *E. coli* produced BMP-2 in urea and NaCl optimally occurred at ranges of 1.0 to 2.0 M urea and 0.75 to 1.25 M NaCl. SDS-PAGE bands of medium intensity were observed within concentration ranges of 0.5 to 1.0 M and 2.0 to 2.5 M urea and 0.5 to 0.75 M and 1.25 to 1.5 M NaCl. Faint bands corresponding to refolded BMP-2 were observed to occur at concentrations in ranges of 0.1 to 0.5 and 2.5 to 3 M urea and 0.25 to 0.5 and 1.5 to 2 M NaCl. Refolding of BMP-2 occurred within the pH range of 7.5 to 11, with better refolding in the pH range of 8.5 to 10.5 and optimal refolding in the pH range of 9 to 10.

Example 5Refolding of *E. coli* Produced BMP-2 in Guanidine/NaCl

- 30 [0057] BMP-2 protein in 1% acetic acid or in reverse phase buffer of 0.1% TFA,, 30-40% acetonitrile was dried or reduced in volume to remove acetonitrile using a speed vacuum, redissolved with four microliters 0.01 % TFA and allowed to dissolve completely for 5 to 10 minutes. A solution containing 8 M to 8.5 M guanidine HCl (guanidine), 100 mM CHES pH 9.5, 5mM EDTA was added to the BMP-2 in TFA and allowed to incubate for 20-30 minutes at room temperature before dilution. The protein concentrations used were such that the final protein concentration in the diluted state was 10 to 100 µg/ml.
- 35 [0058] The guanidine/BMP solution was diluted into a chilled folding buffer (on ice) with the appropriate amount of NaCl and with 50-100 mM CHES pH 9.5, 5 mM EDTA, 2 mM reduced glutathione (GSH), 1 mM oxidized glutathione (GSSG). Samples were argon bubbled (15 seconds) while on ice, and incubated at 4°C.
- 40 [0059] Refolding of the *E. coli* produced BMP-2 in guanidine was analyzed under reducing and non-reducing conditions using Tricine-SDS-PAGE as described above in Example 4.
- [0060] Refolding of *E. coli* produced BMP-2 in guanidine optimally occurred at ranges of 0.18 to 1.0 M guanidine. SDS-PAGE bands of medium intensity were observed within concentration ranges of 0 to 0.18 M and 1.0 to 1.25 M guanidine. Faint bands corresponding to refolded BMP-2 were observed to occur at concentrations in ranges of 1.25 to 1.5M guanidine. Refolding of BMP-2 occurred in guanidine within the pH range of 7.5 to 9.5, with better refolding at pH 8.5 and optimal refolding at pH 9.5. Refolding of BMP-2 was optimal at 4°C, though some refolding was observed at room temperature. (approximately 23°).

Example 6Refolding of *E. coli* BMP-2 in Arginine/NaCl

- 50 [0061] BMP-2 protein in 1% acetic acid or in reverse phase buffer of 0.1 % TFA, 3-40% acetonitrile was dried or reduced in volume to remove acetonitrile using a speed vacuum, redissolved with four microliters of 0.01% TFA and allowed to dissolve completely for 5 to 10 minutes. The protein concentrations used were such that the final protein concentration in the folding buffer was 10 to 100 µg/ml. The folding buffer contained 100 mM buffer titrated to the appropriate pH, 5 mM EDTA, and the desired concentration of salt. Refolding of the *E. coli* produced BMP-2 in arginine was analyzed under reducing and non-reducing conditions using Tricine-SDS-PAGE as described above in Example 4. Substantial bands were observed at all concentrations of arginine used to refold BMP-2; however, the greatest yield

of BMP-2 was obtained using 0.6 to 0.8 M arginine and from 0 to 0.25 M NaCl. Several types of salt were tested for ability to enhance BMP-2 refolding: NaCl, $MgCl_2$, $MgSO_4$, Na_2SO_4 . Of these, NaCl and $MgCl_2$ yielded optimal amounts of refolded BMP-2, and $MgSO_4$ yielded intermediate amounts of refolded BMP-2. The optimal pH range for refolding BMP-2 in arginine is pH 9.5 to 10. Refolding also occurred at pH 8.5. Refolding BMP-2 in arginine was optimal at 4°, though some refolding was observed at room temperature (approximately 23°).

Example 7

Refolding of BMP-2 Using Organic Alcohols

[0062] Denatured, monomeric BMP-2 (and BMP-6) in 1 % acetic acid, prepared as previously described, were added to an Eppendorf tube and lyophilized to dryness. The pellets were redissolved in 20 μ l of 0.01% trifluoroacetic acid. 500 μ l of buffer was then added, containing 50 mM Tris (pH 8.5), 5 mM EDTA, 1.0 M NaCl, 2 mM reduced glutathione, 1 mM oxidized glutathione, and 10-20% methanol, ethanol, or isopropanol. Samples were incubated at room temperature for three days, then evaluated for dimer formation by SDS-PAGE on a 16% Novex tricine gel. A small but discernible amount of BMP-2 dimer was detected after staining with silver. There was no evidence of any BMP-2/6 heterodimer or BMP 6/6 homodimer on the same gels.

Example 8

Purification of Dimeric BMP-2

[0063] Urea refolded BMP-2 protein was injected onto a HPLC C4 analytical column (Vydac 214TP54) equilibrated to 10% B buffer (A buffer = 0.1 % TFA, B buffer = 95% acetonitrile, 0.1% TFA), with a flow rate of 1 ml/min. After 15 minutes, a linear gradient from 10% to 50% B buffer was applied over 40 minutes, during which time the dimeric BMP-2 protein eluted. Protein was monitored by absorbance at 280 nm. Peak BMP-2 dimer fractions (eluting between 45 and 48 minutes) were pooled, analyzed by 16% Tricine-SDS-PAGE, and tested for biological activity in the assays described in Examples 9 and 10.

Example 9

W-20 Alkaline Phosphatase Assay Protocol

[0064] W-20-17 cells are plated into 96 well tissue culture plates at a density of 10,000 cells per well in 200 μ l of medium (DME with 10% heat inactivated fetal calf serum, 2 mM glutamine). The cells are allowed to attach overnight in a 95 % air, 5 % CO_2 incubator at 37°C.

[0065] The 200 μ l of medium is removed from each well with a multichannel pipettor and replaced with an equal volume of test sample delivered in DME with 10% heat inactivated fetal calf serum, 2 mM glutamine and 1 % penicillin-streptomycin.

[0066] The test samples and standards are allowed a 24 hour incubation period with the W-20-17 indicator cells. After the 24 hours, plates are removed from the 37°C incubator and the test media are removed from the cells.

[0067] The W-20-17 cell layers are washed three times with 200 μ l per well of calcium/magnesium free phosphate buffered saline and these washes are discarded.

[0068] 50 μ l of glass distilled water is added to each well and the assay plates are then placed on a dry ice/ethanol bath for quick freezing. Once frozen, the assay plates are removed from the dry ice/ethanol bath and thawed at 37°C. This step is repeated two more times for a total of 3 freeze-thaw procedures. Once complete, the membrane bound alkaline phosphatase is available for measurement.

[0069] 50 μ l of assay mix (50 mM glycine, 0.05% Triton X-100, 4 mM $MgCl_2$, 5 mM p-nitrophenol phosphate, pH = 10.3) is added to each assay well and the assay plates are then incubated for 30 minutes at 37°C in a shaking waterbath at 60 oscillations per minute.

[0070] At the end of the 30 minute incubation, the reaction is stopped by adding 100 μ l of 0.2 N NaOH to each well and placing the assay plates on ice.

[0071] The spectrophotometric absorbance for each well is read at a wavelength of 405 nanometers. These values are then compared to known standards to give an estimate of the alkaline phosphatase activity in each sample. For example, using known amounts of p-nitrophenol phosphate, absorbance values are generated. This is shown in Table I.

Table I

Absorbance Values for Known Standards of P-Nitrophenol Phosphate	
P-nitrophenol Phosphate moles	Mean Absorbance (405 nm)
0.000	0
0.006	0.261 +/- .024
0.012	0.521 +/- .031
0.018	0.797 +/- .063
0.024	1.074 +/- .061
0.030	1.305 +/- .083

[0072] Absorbance values for known amounts of BMP-2 can be determined and converted to moles of p-nitrophenol phosphate cleaved per unit time as shown in Table II.

Table II

Alkaline Phosphatase Values for W-20 Cells Treated with BMP-2		
BMP-2 concentration ng/ml	Absorbance Reading 405 nmeters	umoles substrate per hour
0	0.645	0.024
1.56	0.696	0.026
3.12	0.765	0.029
6.25	0.923	0.036
12.50	1.121	0.044
25.0	1.457	0.058
50.0	1.662	0.067
100.0	1.977	0.08

[0073] These values are then used to compare the activities of known amounts of BMP heterodimers to BMP-2 homodimer.

Example 10

Rosen-Modified Sampath-Reddi Assay

[0074] The ethanol precipitation step of the Sampath-Reddi procedure, *supra*, is replaced by dialyzing (if the composition is a solution) or diafiltering (if the composition is a suspension) the fraction to be assayed against water. The solution or suspension is then redissolved in 0.1 % TFA, and the resulting solution added to 20 mg of rat matrix. A mock rat matrix sample not treated with the protein serves as a control. This material is frozen and lyophilized and the resulting powder enclosed in #5 gelatin capsules. The capsules are implanted subcutaneously in the abdominal thoracic area of 21-49 day old male Long Evans rats. The implants are removed after 7-14 days. Half of each implant is used for alkaline phosphatase analysis [see, A. H. Reddi, et al., *Proc. Natl. Acad. Sci.*, 69:1601 (1972)]

[0075] The other half of each implant is fixed and processed for histological analysis. One μ m glycolmethacrylate sections are stained with Von Kossa and acid fuchsin to score the amount of induced bone and cartilage formation present in each implant. The terms +1 through +5 represent the area of each histological section of an implant occupied by new bone and/or cartilage cells and matrix. A score of +5 indicates that greater than 50% of the implant is new bone and/or cartilage produced as a direct result of protein in the implant. A score of +4, +3, +2, and +1 would indicate that greater than 40%, 30%, 20% and 10% respectively of the implant contains new cartilage and/or bone.

SEQUENCE LISTING

[0076]

5 (1) GENERAL INFORMATION:

(i) APPLICANT: GENETICS INSTITUTE, INC.

10 (ii) TITLE OF INVENTION: MUTANTS OF BONE MORPHOGENIC PROTEINS

(iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDENCE ADDRESS:

15 (A) ADDRESSEE: Genetics Institute, Inc - Legal Affairs

(B) STREET: 87 CambridgePark Drive

(C) CITY: Cambridge

(D) STATE: Massachusetts

(E) COUNTRY: USA

20 (F) ZIP: 02140

(v) COMPUTER READABLE FORM:

25 (A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: Patent In Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

30

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

35 (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/163,877

(B) FILING DATE: December 7, 1993

40 (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Lazar, Steven R.

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45 (C) REFERENCE/DOCKET NUMBER: GI 5219-PCT

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(A) TELEPHONE: 617 498-8260

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50

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

55 (A) LENGTH: 342 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: unknown

EP 0 733 108 B1

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: bmp-2

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..342

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

15	CAA GCC AAA CAC AAA CAG CGG AAA CGC CTT AAG TCC AGC TGT AAG AGA	48
	Gln Ala Lys His Lys Gln Arg Lys Arg Leu Lys Ser Ser Cys Lys Arg	
	1 5 10 15	
	CAC CCT TTG TAC GTG GAC TTC AGT GAC GTG GGG TGG AAT GAC TGG ATT	96
	His Pro Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp Trp Ile	
	20 25 30	
25	GTG GCT CCC CCG GGG TAT CAC GCC TTT TAC TGC CAC GGA GAA TGC CCT	144
	Val Ala Pro Pro Gly Tyr His Ala Phe Tyr Cys His Gly Glu Cys Pro	
	35 40 45	
	TTT CCT CTG GCT GAT CAT CTG AAC TCC ACT AAT CAT GCC ATT GTT CAG	192
	Phe Pro Leu Ala Asp His Leu Asn Ser Thr Asn His Ala Ile Val Gln	
	50 55 60	
30	ACG TTG GTC AAC TCT GTT AAC TCT AAG ATT CCT AAG GCA TGC TGT GTC	240
	Thr Leu Val Asn Ser Val Asn Ser Lys Ile Pro Lys Ala Cys Cys Val	
	65 70 75 80	
35	CCG ACA GAA CTC AGT GCT ATC TCG ATG CTG TAC CTT GAC GAG AAT GAA	288
	Pro Thr Glu Leu Ser Ala Ile Ser Met Leu Tyr Leu Asp Glu Asn Glu	
	85 90 95	
	AAG GTT GTA TTA AAG AAC TAT CAG GAC ATG GTT GTG GAG GGT TGT GGG	336
	Lys Val Val Leu Lys Asn Tyr Gln Asp Met Val Val Glu Gly Cys Gly	
	100 105 110	
40	TGT CGC	342
	Cys Arg	

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 114 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

EP 0 733 108 B1

1 Gln Ala Lys His Lys Gln Arg Lys Arg Leu Lys Ser Ser Cys Lys Arg
 5 His Pro Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp Trp Ile
 10 Val Ala Pro Pro Gly Tyr His Ala Phe Tyr Cys His Gly Glu Cys Pro
 15 Phe Pro Leu Ala Asp His Leu Asn Ser Thr Asn His Ala Ile Val Gln
 20 Thr Leu Val Asn Ser Val Asn Ser Lys Ile Pro Lys Ala Cys Cys Val
 25 Pro Thr Glu Leu Ser Ala Ile Ser Met Leu Tyr Leu Asp Glu Asn Glu
 30 Lys Val Val Leu Lys Asn Tyr Gln Asp Met Val Val Glu Gly Cys Gly
 35 Cys Arg

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 348 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: bmp-4

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..348

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

EP 0 733 108 B1

AGC CCT AAG CAT CAC TCA CAG CGG GCC AGG AAG AAG AAT AAG AAC TGC 48
 Ser Pro Lys His His Ser Gln Arg Ala Arg Lys Lys Asn Lys Asn Cys
 1 5 10 15

5 CGG CGC CAC TCG CTC TAT GTG GAC TTC AGC GAT GTG GGC TGG AAT GAC 96
 Arg Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp
 20 25 30

10 TGG ATT GTG GCC CCA CCA GGC TAC CAG GCC TTC TAC TGC CAT GGG GAC 144
 Trp Ile Val Ala Pro Pro Gly Tyr Gln Ala Phe Tyr Cys His Gly Asp
 35 40 45

TGC CCC TTT CCA CTG GCT GAC CAC CTC AAC TCA ACC AAC CAT GCC ATT 192
 Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr Asn His Ala Ile
 50 55 60

15 GTG CAG ACC CTG GTC AAT TCT GTC AAT TCC AGT ATC CCC AAA GCC TGT 240
 Val Gln Thr Leu Val Asn Ser Val Asn Ser Ser Ile Pro Lys Ala Cys
 65 70 75 80

20 TGT GTG CCC ACT GAA CTG AGT GCC ATC TCC ATG CTG TAC CTG GAT GAG 288
 Cys Val Pro Thr Glu Leu Ser Ala Ile Ser Met Leu Tyr Leu Asp Glu
 85 90 95

TAT GAT AAG GTG GTA CTG AAA AAT TAT CAG GAG ATG GTA GTA GAG GGA 336
 Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu Met Val Val Glu Gly
 100 105 110

25 TGT GGG TGC CGC 348
 Cys Gly Cys Arg
 115

(2) INFORMATION FOR SEQ ID NO:4:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 116 amino acids

(B) TYPE: amino acid

35

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

40

Ser Pro Lys His His Ser Gln Arg Ala Arg Lys Lys Asn Lys Asn Cys
 1 5 10 15

45

Arg Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp
 20 25 30

Trp Ile Val Ala Pro Pro Gly Tyr Gln Ala Phe Tyr Cys His Gly Asp
 35 40 45

50

Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr Asn His Ala Ile
 50 55 60

55

EP 0 733 108 B1

Val Gln Thr Leu Val Asn Ser Val Asn Ser Ser Ile Pro Lys Ala Cys
65 70 75 80
5 Cys Val Pro Thr Glu Leu Ser Ala Ile Ser Met Leu Tyr Leu Asp Glu
85 90 95
Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu Met Val Val Glu Gly
100 105 110
10 Cys Gly Cys Arg
115

(2) INFORMATION FOR SEQ ID NO:5:

15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 396 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

25 (A) ORGANISM: bmp-5

(ix) FEATURE:

(A) NAME/KEY: CDS
30 (B) LOCATION: 1..396

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

35

40

45

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55

EP 0 733 108 B1

	AAT CAA AAC CGC AAT AAA TCC AGC TCT CAT CAG GAC TCC TCC AGA ATG	48
	Asn Gln Asn Arg Asn Lys Ser Ser Ser His Gln Asp Ser Ser Arg Met	
	1 5 10 15	
5	TCC AGT GTT GGA GAT TAT AAC ACA AGT GAG CAA AAA CAA GCC TGT AAG	96
	Ser Ser Val Gly Asp Tyr Asn Thr Ser Glu Gln Lys Gln Ala Cys Lys	
	20 25 30	
10	AAG CAC GAA CTC TAT GTG AGC TTC CGG GAT CTG GGA TGG CAG GAC TGG	144
	Lys His Glu Leu Tyr Val Ser Phe Arg Asp Leu Gly Trp Gln Asp Trp	
	35 40 45	
15	ATT ATA GCA CCA GAA GGA TAC GCT GCA TTT TAT TGT GAT GGA GAA TGT	192
	Ile Ile Ala Pro Glu Gly Tyr Ala Ala Phe Tyr Cys Asp Gly Glu Cys	
	50 55 60	
20	TCT TTT CCA CTT AAC GCC CAT ATG AAT GCC ACC AAC CAC GCT ATA GTT	240
	Ser Phe Pro Leu Asn Ala His Met Asn Ala Thr Asn His Ala Ile Val	
	65 70 75 80	
25	CAG ACT CTG GTT CAT CTG ATG TTT CCT GAC CAC GTA CCA AAG CCT TGT	288
	Gln Thr Leu Val His Leu Met Phe Pro Asp His Val Pro Lys Pro Cys	
	85 90 95	
30	TGT GCT CCA ACC AAA TTA AAT GCC ATC TCT GTT CTG TAC TTT GAT GAC	336
	Cys Ala Pro Thr Lys Leu Asn Ala Ile Ser Val Leu Tyr Phe Asp Asp	
	100 105 110	
35	AGC TCC AAT GTC ATT TTG AAA AAA TAT AGA AAT ATG GTA GTA CGC TCA	384
	Ser Ser Asn Val Ile Leu Lys Lys Tyr Arg Asn Met Val Val Arg Ser	
	115 120 125	
40	TGT GGC TGC CAC	396
	Cys Gly Cys His	
	130	

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 132 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Asn Gln Asn Arg Asn Lys Ser Ser Ser His Gln Asp Ser Ser Arg Met
 1 5 10 15
 5 Ser Ser Val Gly Asp Tyr Asn Thr Ser Glu Gln Lys Gln Ala Cys Lys
 20 25 30
 Lys His Glu Leu Tyr Val Ser Phe Arg Asp Leu Gly Trp Gln Asp Trp
 35 40 45
 10 Ile Ile Ala Pro Glu Gly Tyr Ala Ala Phe Tyr Cys Asp Gly Glu Cys
 50 55 60
 Ser Phe Pro Leu Asn Ala His Met Asn Ala Thr Asn His Ala Ile Val
 65 70 75 80
 15 Gln Thr Leu Val His Leu Met Phe Pro Asp His Val Pro Lys Pro Cys
 85 90 95
 Cys Ala Pro Thr Lys Leu Asn Ala Ile Ser Val Leu Tyr Phe Asp Asp
 100 105 110
 20 Ser Ser Asn Val Ile Leu Lys Lys Tyr Arg Asn Met Val Val Arg Ser
 115 120 125
 Cys Gly Cys His
 130

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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 406 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: bmp-6

40 (ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 1..396

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAA CAG AGT CGT AAT CGC TCT ACC CAG TCC CAG GAC GTG GCG CGG GTC 48
 Gln Gln Ser Arg Asn Arg Ser Thr Gln Ser Gln Asp Val Ala Arg Val
 1 5 10 15
 TCC AGT GCT TCA GAT TAC AAC AGC AGT GAA TTG AAA ACA GCC TGC AGG 96
 Ser Ser Ala Ser Asp Tyr Asn Ser Ser Glu Leu Lys Thr Ala Cys Arg
 20 25 30

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	AAG CAT GAG CTG TAT GTG AGT TTC CAA GAC CTG GGA TGG CAG GAC TGG	144
	Lys His Glu Leu Tyr Val Ser Phe Gln Asp Leu Gly Trp Gln Asp Trp	
	35 40 45	
5	ATC ATT GCA CCC AAG GGC TAT GCT GCC AAT TAC TGT GAT GGA GAA TGC	192
	Ile Ile Ala Pro Lys Gly Tyr Ala Ala Asn Tyr Cys Asp Gly Glu Cys	
	50 55 60	
10	TCC TTC CCA CTC AAC GCA CAC ATG AAT GCA ACC AAC CAC GCG ATT GTG	240
	Ser Phe Pro Leu Asn Ala His Met Asn Ala Thr Asn His Ala Ile Val	
	65 70 75 80	
	CAG ACC TTG GTT CAC CTT ATG AAC CCC GAG TAT GTC CCC AAA CCG TGC	288
	Gln Thr Leu Val His Leu Met Asn Pro Glu Tyr Val Pro Lys Pro Cys	
	85 90 95	
15	TGT GCG CCA ACT AAG CTA AAT GCC ATC TCG GTT CTT TAC TTT GAT GAC	336
	Cys Ala Pro Thr Lys Leu Asn Ala Ile Ser Val Leu Tyr Phe Asp Asp	
	100 105 110	
20	AAC TCC AAT GTC ATT CTG AAA AAA TAC AGG AAT ATG GTT GTA AGA GCT	384
	Asn Ser Asn Val Ile Leu Lys Lys Tyr Arg Asn Met Val Val Arg Ala	
	115 120 125	
	TGT GGA TGC CAC TAACTCGAAA	406
	Cys Gly Cys His	
	130	

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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 132 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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1 Gln Gln Ser Arg Asn Arg Ser Thr Gln Ser Gln Asp Val Ala Arg Val
 5 Ser Ser Ala Ser Asp Tyr Asn Ser Ser Glu Leu Lys Thr Ala Cys Arg
 10 Lys His Glu Leu Tyr Val Ser Phe Gln Asp Leu Gly Trp Gln Asp Trp
 15 Ile Ile Ala Pro Lys Gly Tyr Ala Ala Asn Tyr Cys Asp Gly Glu Cys
 20 Ser Phe Pro Leu Asn Ala His Met Asn Ala Thr Asn His Ala Ile Val
 25 Gln Thr Leu Val His Leu Met Asn Pro Glu Tyr Val Pro Lys Pro Cys
 30 Cys Ala Pro Thr Lys Leu Asn Ala Ile Ser Val Leu Tyr Phe Asp Asp
 35 Asn Ser Asn Val Ile Leu Lys Lys Tyr Arg Asn Met Val Val Arg Ala
 40 Cys Gly Cys His
 45
 50
 55

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 417 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: bmp-7

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..417

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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	TCC	ACG	GGG	AGC	AAA	CAG	CGC	AGC	CAG	AAC	CGC	TCC	AAG	ACG	CCC	AAG	48
	Ser	Thr	Gly	Ser	Lys	Gln	Arg	Ser	Gln	Asn	Arg	Ser	Lys	Thr	Pro	Lys	
	1				5					10					15		
5	AAC	CAG	GAA	GCC	CTG	CGG	ATG	GCC	AAC	GTG	GCA	GAG	AAC	AGC	AGC	AGC	96
	Asn	Gln	Glu	Ala	Leu	Arg	Met	Ala	Asn	Val	Ala	Glu	Asn	Ser	Ser	Ser	
				20					25					30			
	GAC	CAG	AGG	CAG	GCC	TGT	AAG	AAG	CAC	GAG	CTG	TAT	GTC	AGC	TTC	CGA	144
10	Asp	Gln	Arg	Gln	Ala	Cys	Lys	Lys	His	Glu	Leu	Tyr	Val	Ser	Phe	Arg	
				35				40					45				
	GAC	CTG	GGC	TGG	CAG	GAC	TGG	ATC	ATC	GCG	CCT	GAA	GGC	TAC	GCC	GCC	192
	Asp	Leu	Gly	Trp	Gln	Asp	Trp	Ile	Ile	Ala	Pro	Glu	Gly	Tyr	Ala	Ala	
				50			55					60					
15	TAC	TAC	TGT	GAG	GGG	GAG	TGT	GCC	TTC	CCT	CTG	AAC	TCC	TAC	ATG	AAC	240
	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ala	Phe	Pro	Leu	Asn	Ser	Tyr	Met	Asn	
	65				70					75					80		
	GCC	ACC	AAC	CAC	GCC	ATC	GTG	CAG	ACG	CTG	GTC	CAC	TTC	ATC	AAC	CCG	288
20	Ala	Thr	Asn	His	Ala	Ile	Val	Gln	Thr	Leu	Val	His	Phe	Ile	Asn	Pro	
				85						90					95		
	GAA	ACG	GTG	CCC	AAG	CCC	TGC	TGT	GCG	CCC	ACG	CAG	CTC	AAT	GCC	ATC	336
	Glu	Thr	Val	Pro	Lys	Pro	Cys	Cys	Ala	Pro	Thr	Gln	Leu	Asn	Ala	Ile	
				100					105					110			
25	TCC	GTC	CTC	TAC	TTC	GAT	GAC	AGC	TCC	AAC	GTC	ATC	CTG	AAG	AAA	TAC	384
	Ser	Val	Leu	Tyr	Phe	Asp	Asp	Ser	Ser	Asn	Val	Ile	Leu	Lys	Lys	Tyr	
				115				120					125				
	AGA	AAC	ATG	GTG	GTC	CGG	GCC	TGT	GGC	TGC	CAC						417
30	Arg	Asn	Met	Val	Val	Arg	Ala	Cys	Gly	Cys	His						
				130			135										

(2) INFORMATION FOR SEQ ID NO:10:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 139 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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Ser Thr Gly Ser Lys Gln Arg Ser Gln Asn Arg Ser Lys Thr Pro Lys
 1 5 10 15
 5 Asn Gln Glu Ala Leu Arg Met Ala Asn Val Ala Glu Asn Ser Ser Ser
 20 25 30
 Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr Val Ser Phe Arg
 35 40 45
 10 Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu Gly Tyr Ala Ala
 50 55 60
 Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn Ser Tyr Met Asn
 65 70 75 80
 15 Ala Thr Asn His Ala Ile Val Gln Thr Leu Val His Phe Ile Asn Pro
 85 90 95
 Glu Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln Leu Asn Ala Ile
 100 105 110
 20 Ser Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile Leu Lys Lys Tyr
 115 120 125
 Arg Asn Met Val Val Arg Ala Cys Gly Cys His
 130 135

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(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

30

- (A) LENGTH: 420 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(B) STRAIN: BMP-8

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(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..417

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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	GCA GTG AGG CCA CTG AGG AGG AGG CAG CCG AAG AAA AGC AAC GAG CTG	48
	Ala Val Arg Pro Leu Arg Arg Arg Gln Pro Lys Lys Ser Asn Glu Leu	
	1 5 10 15	
5	CCG CAG GCC AAC CGA CTC CCA GGG ATC TTT GAT GAC GTC CAC GGC TCC	96
	Pro Gln Ala Asn Arg Leu Pro Gly Ile Phe Asp Asp Val His Gly Ser	
	20 25 30	
10	CAC GGC CGG CAG GTC TGC CGT CGG CAC GAG CTC TAC GTC AGC TTC CAG	144
	His Gly Arg Gln Val Cys Arg Arg His Glu Leu Tyr Val Ser Phe Gln	
	35 40 45	
	GAC CTT GGC TGG CTG GAC TGG GTC ATC GCC CCC CAA GGC TAC TCA GCC	192
	Asp Leu Gly Trp Leu Asp Trp Val Ile Ala Pro Gln Gly Tyr Ser Ala	
	50 55 60	
15	TAT TAC TGT GAG GGG GAG TGC TCC TTC CCG CTG GAC TCC TGC ATG AAC	240
20	Tyr Tyr Cys Glu Gly Glu Cys Ser Phe Pro Leu Asp Ser Cys Met Asn	
	65 70 75 80	
	GCC ACC AAC CAC GCC ATC CTG CAG TCC CTG GTG CAC CTG ATG AAG CCA	288
	Ala Thr Asn His Ala Ile Leu Gln Ser Leu Val His Leu Met Lys Pro	
25	85 90 95	
	AAC GCA GTC CCC AAG GCG TGC TGT GCA CCC ACC AAG CTG AGC GCC ACC	336
	Asn Ala Val Pro Lys Ala Cys Cys Ala Pro Thr Lys Leu Ser Ala Thr	
	100 105 110	
30	TCT GTG CTC TAC TAT GAC AGC AGC AAC AAC GTC ATC CTG CGC AAG CAC	384
	Ser Val Leu Tyr Tyr Asp Ser Ser Asn Asn Val Ile Leu Arg Lys His	
	115 120 125	
	CGC AAC ATG GTG GTC AAG GCC TGC GGC TGC CAC TGA	420
35	Arg Asn Met Val Val Lys Ala Cys Gly Cys His	
	130 135	

(2) INFORMATION FOR SEQ ID NO:12:

40 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 139 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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Ala Val Arg Pro Leu Arg Arg Arg Gln Pro Lys Lys Ser Asn Glu Leu
 1 5 10 15
 5 Pro Gln Ala Asn Arg Leu Pro Gly Ile Phe Asp Asp Val His Gly Ser
 20 25 30
 His Gly Arg Gln Val Cys Arg Arg His Glu Leu Tyr Val Ser Phe Gln
 35 40 45
 10 Asp Leu Gly Trp Leu Asp Trp Val Ile Ala Pro Gln Gly Tyr Ser Ala
 50 55 60
 Tyr Tyr Cys Glu Gly Glu Cys Ser Phe Pro Leu Asp Ser Cys Met Asn
 65 70 75 80
 15 Ala Thr Asn His Ala Ile Leu Gln Ser Leu Val His Leu Met Lys Pro
 85 90 95
 Asn Ala Val Pro Lys Ala Cys Cys Ala Pro Thr Lys Leu Ser Ala Thr
 100 105 110
 20 Ser Val Leu Tyr Tyr Asp Ser Ser Asn Asn Val Ile Leu Arg Lys His
 115 120 125
 Arg Asn Met Val Val Lys Ala Cys Gly Cys His
 130 135

Claims

- 30 1. A recombinant DNA molecule encoding a mutant bone morphogenetic protein (BMP) comprising:
 (a) a nucleotide sequence encoding BMP-4 as shown in SEQ ID NO:3, except that the nucleotide triplet at 319 to 321 has been replaced with a triplet encoding aspartic acid;
 (b) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:4, wherein the glutamic acid at
 35 amino acid residue 107 has been replaced with aspartic acid;
 (c) a nucleotide sequence encoding BMP-5 as shown in SEQ-ID NO:5, except that the nucleotide triplet at 166 to 168 has been replaced with a triplet encoding histidine;
 (d) a nucleotide sequence encoding the amino acid sequences of SEQ ID NO:6, wherein the alanine at amino
 40 acid residue 56 has been replaced with histidine;
 (e) a nucleotide sequence encoding BMP-6 as shown in SEQ ID NO:7, except that the nucleotide triplet at 166 to 168 has been replaced with a triplet encoding histidine;
 (f) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:8, wherein the alanine at amino
 acid residue 56 has been replaced with histidine;
 (g) a nucleotide sequence encoding BMP-7 as shown in SEQ ID NO:9, except that the nucleotide triplet at
 45 187 to 189 has been replaced with a triplet encoding histidine;
 (h) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:10, wherein the alanine at amino
 acid residue 63 has been replaced with histidine;
 (i) a nucleotide sequence encoding BMP-8 as shown in SEQ ID NO:11, except that the nucleotide triplet at
 187 to 189 has been replaced with a triplet encoding histidine; or
 50 (j) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO:12, wherein the amino acid residue 63 has been replaced with histidine.
2. A plasmid containing the recombinant DNA molecule of claim 1.
- 55 3. A bacterial cell transformed with the plasmid of claim 2.
4. A method of producing a mutant BMP, comprising:

- (a) culturing the bacterial cell of claim 3; and
- (b) recovering the mutant BMP from the culture medium.

5. A mutant BMP encoded by the recombinant DNA molecule of claim 1.

6. A BMP heterodimer comprising at least one mutant BMP of claim 5.

7. A method of obtaining mutants of bone morphogenetic proteins (BMP) with improved refolding properties, said method comprising:

- (a) comparing the amino acid sequence of a BMP which is found to refold well (BMP+) with the amino acid sequence of a BMP which does not refold well (BMP-);
- (b) determining the differences at correlative amino acid positions in the comparison of step (a);
- (c) altering the amino acid sequence of BMP- so that one amino acid which is different from that of the correlative amino acid of BMP+ is replaced by the correlative amino acid of BMP+ to form a modified BMP- protein;
- (d) testing the modified BMP- protein for its ability to refold.

8. A pharmaceutical composition comprising the mutant BMP of claim 5 or the BMP heterodimer of claim 6.

9. Use of the mutant BMP of claim 5 or the BMP heterodimer of claim 6 for the preparation of a composition for the treatment of bone or cartilage injuries or disorders.

Patentansprüche

1. Rekombinantes DNA-Molekül, das ein mutiertes morphogenetisches Knochenprotein (BMP) codiert, umfassend:

(a) eine Nucleotidsequenz wie in SEQ ID Nr: 3 gezeigt, die BMP-4 codiert, mit der Ausnahme, daß das Nucleotidtriplett von 319 bis 321 durch ein Triplett ersetzt ist, das Asparaginsäure codiert;

(b) eine Nucleotidsequenz, die die Aminosäuresequenz von SEQ ID Nr: 4 codiert, worin die Glutaminsäure am Aminosäurerest 107 durch Asparaginsäure ersetzt ist;

(c) eine Nucleotidsequenz wie in SEQ ID Nr: 5 gezeigt, die BMP-5 codiert, mit der Ausnahme, daß das Nucleotidtriplett von 166 bis 168 durch ein Triplett ersetzt ist, das Histidin codiert;

(d) eine Nucleotidsequenz, die die Aminosäuresequenz von SEQ ID Nr: 6 codiert, worin das Alanin am Aminosäurerest 56 durch Histidin ersetzt ist;

(e) eine Nucleotidsequenz wie in SEQ ID Nr: 7 gezeigt, die BMP-6 codiert, mit der Ausnahme, daß das Nucleotidtriplett von 166 bis 168 durch ein Triplett ersetzt ist, das Histidin codiert;

(f) eine Nucleotidsequenz, die die Aminosäuresequenz von SEQ ID Nr: 8 codiert, worin das Alanin am Aminosäurerest 56 durch Histidin ersetzt ist;

(g) eine Nucleotidsequenz wie in SEQ ID Nr: 9 gezeigt, die BMP-7 codiert, mit der Ausnahme, daß das Nucleotidtriplett von 187 bis 189 durch ein Triplett ersetzt ist, das Histidin codiert;

(h) eine Nucleotidsequenz, die die Aminosäuresequenz von SEQ ID Nr: 10 codiert, worin das Alanin am Aminosäurerest 63 durch Histidin ersetzt ist;

(i) eine Nucleotidsequenz wie in SEQ ID Nr: 11 gezeigt, die BMP-8 codiert, mit der Ausnahme, daß das Nucleotidtriplett von 187 bis 189 durch ein Triplett ersetzt ist, das Histidin codiert; oder

(j) eine Nucleotidsequenz, die die Aminosäuresequenz von SEQ ID Nr: 12 codiert, worin der Aminosäurerest 63 durch Histidin ersetzt ist.

2. Plasmid, das das rekombinante DNA-Molekül nach Anspruch 1 enthält.

3. Bakterienzelle, die mit dem Plasmid nach Anspruch 2 transformiert ist.
4. Verfahren zur Herstellung von mutiertem BMP, umfassend:
 - 5 (a) Züchtung der Bakterienzelle nach Anspruch 3; und
 - (b) Gewinnung des mutierten BMPs aus dem Kulturmedium.
5. Mutiertes BMP, das von dem rekombinanten DNA-Molekül nach Anspruch 1 codiert wird.
- 10 6. BMP-Heterodimer, das mindestens ein mutiertes BMP nach Anspruch 5 umfaßt.
7. Verfahren zur Gewinnung von mutierten morphogenetischen Knochenproteinen (BMP) mit verbesserten Rückfaltungseigenschaften, das umfasst:
 - 15 (a) Vergleich der Aminosäuresequenz eines BMP, von dem gezeigt wurde, daß es sich gut zurückfaltet (BMP+), mit der Aminosäuresequenz eines BMP, welches sich nicht gut zurückfaltet (BMP-);
 - (b) Bestimmung der Unterschiede an den entsprechenden Aminosäure-Positionen im Vergleich von Schritt (a);
 - 20 (c) Veränderung der Aminosäuresequenz von BMP-, so daß eine Aminosäure, die sich von der entsprechenden Aminosäure von BMP+ unterscheidet, durch die entsprechende Aminosäure aus BMP+ ersetzt wird, um ein modifiziertes BMP- Protein zu erzeugen;
 - 25 (d) Testen des modifizierten BMP- Proteins bezüglich seiner Rückfaltungsfähigkeit.
8. Arzneimittel, umfassend das mutierte BMP nach Anspruch 5 oder das BMP-Heterodimer nach Anspruch 6.
9. Verwendung des mutierten BMP nach Anspruch 5 oder des BMP-Heterodimers nach Anspruch 6 für die Herstellung einer Zusammensetzung zur Behandlung von Knochen- oder Knorpelverletzungen oder -erkrankungen.
- 30

Revendications

- 35 1. Molécule d'ADN recombinant, codant pour une protéine morphogénétique osseuses mutante (BMP), comprenant :
 - (a) une séquence nucléotidique codant pour BMP-4, telle que représentée dans SEQ ID n° 3, mis à part que le triplet de nucléotides à la position 319 à 321 a été remplacé par un triplet codant pour l'acide aspartique;
 - 40 (b) une séquence nucléotidique codant pour la séquence d'acides aminés de SEQ ID n° 4, dans laquelle l'acide glutamique au niveau du résidu acide aminé 107 a été remplacé par l'acide aspartique;
 - (c) une séquence nucléotidique codant pour BMP-5, telle que représentée dans SEQ ID n° 5, mis à part que le triplet de nucléotides à la position 166 à 168 a été remplacé par un triplet codant pour l'histidine ;
 - (d) une séquence nucléotidique codant pour la séquence d'acides aminés de SEQ ID n° 6, dans laquelle l'alanine au niveau du résidu acide aminé 56 a été remplacée par l'histidine ;
 - 45 (e) une séquence nucléotidique codant pour BMP-6, telle que représentée dans SEQ ID n° 7, mis à part que le triplet de nucléotides à la position 166 à 168 a été remplacé par un triplet codant pour l'histidine ;
 - (f) une séquence nucléotidique codant pour la séquence d'acides aminés de SEQ ID n° 8, dans laquelle l'alanine au niveau du résidu acide aminé 56 a été remplacée par l'histidine ;
 - (g) une séquence nucléotidique codant pour BMP-7, telle que représentée dans SEQ ID n° 9, mis à part que le triplet de nucléotides à la position 187 à 189 a été remplacé par un triplet codant pour l'histidine ;
 - 50 (h) une séquence nucléotidique codant pour la séquence d'acides aminés de SEQ ID n° 10, dans laquelle l'alanine au niveau du résidu acide aminé 63 a été remplacée par l'histidine ;
 - (i) une séquence nucléotidique codant pour BMP-8, telle que représentée dans SEQ ID n° 11, mis à part que le triplet de nucléotides à la position 187 à 189 a été remplacé par un triplet codant pour l'histidine ; ou
 - 55 (j) une séquence nucléotidique codant pour la séquence d'acides aminés de SEQ ID n° 12, dans laquelle le résidu acide aminé 63 a été remplacé par l'histidine ;
2. Plasmide contenant la molécule d'ADN recombinant de la revendication 1.

3. Cellule bactérienne transformée par le plasmide de la revendication 2.
4. Procédé de production d'une BMP mutante, comprenant :
 - 5 (a) la culture de la cellule bactérienne de la revendication 3 ; et
(b) la récupération de la BMP mutante à partir du milieu de culture.
5. BMP mutante codée par la molécule d'ADN recombinant de la revendication 1.
- 10 6. Hétérodimère de BMP comprenant au moins une BMP mutante de la revendication 5.
7. Procédé d'obtention de mutants de protéines morphogénétiques osseuses (BMP) à propriétés de repliement améliorées, ledit procédé comprenant :
 - 15 (a) la comparaison de la séquence d'acides aminés d'une BMP qui se révèle se replier de façon satisfaisante (BMP+) avec la séquence d'acides aminés d'une BMP qui ne se replie pas de façon satisfaisante (BMP-);
(b) la détermination des différences au niveau de positions d'acides aminés corrélatifs dans la comparaison de l'étape (a) ;
(c) la modification de la séquence d'acides aminés de BMP- de manière qu'un acide aminé qui est différent de
20 celui de l'acide aminé corrélatif de BMP+ soit remplacé par l'acide aminé corrélatif de BMP+, pour former une protéine BMP- modifiée;
(d) l'essai de la protéine BMP- modifiée pour déterminer son aptitude au repliement.
8. Composition pharmaceutique comprenant la BMP mutante de la revendication 5 ou l'hétérodimère de BMP de la
25 revendication 6.
9. Utilisation de la BMP mutante de la revendication 5 ou de l'hétérodimère de BMP de la revendication 6 pour la
30 préparation d'une composition destinée au traitement de troubles ou de lésions de l'os ou du cartilage.

COMPARISON OF BMP SEQUENCES

BMP4					SP	2
BMP5				NQNRNKSSSH	QDSSRMS	17
BMP6				QQSRNRSTQS	QDVARVS	17
BMP7			STGSKQR	SQNRSKTPKN	QEALRMA	24
BMP2	QAKHKQRKRL	KSS CKRHPLY	VDFSDVGWND	WIVAPPGYHA	FYCHGECPPP	50
BMP4	KHHSQRARKK	NKNCRRHSLY	VDFSDVGWND	WIVAPPGYQA	FYCHGDCPPF	52
BMP5	SVGDYNTS BQ	KQACKKHHLT	VSFRDLGWQD	WII APEGYAA	FYCDGECSPF	67
BMP6	SAS DYNSS EL	KTACKKHHLT	VSFQDLGWQD	WII APKGYAA	NYCDGECSPF	67
BMP7	NVAENSS SDQ	RQACKKHELY	VSFRDLGWQD	WII APEGYAA	YYCEGECAPF	74
BMP2	LADHL NSTNH	AIVQTLVNSV	NSK.. I PKAC	CVPTLS AIS	MLYLDENEKV	98
BMP4	LADHL NSTNH	AIVQTLVNSV	NSS.. I PKAC	CVPTLS AIS	MLYLDEYDKV	100
BMP5	LNAHMNATNH	AIVQTLVHLM	FPDH.VPKPC	CAPTQLNAIS	VLYF DDSSNV	116
BMP6	LNAHMNATNH	AIVQTLVHLM	NPEY.VPKPC	CAPTQLNAIS	VLYF DDSSNV	116
BMP7	LNSYMNATNH	AIVQTLVHFI	NPET.VPKPC	CAPTQLNAIS	VLYF DDSSNV	123
BMP2	VLKQYQDMVV	EG. CGCR				114
BMP4	VLKQYQDMVV	EG. CGCR				116
BMP5	ILKKYRNMVV	RS. CGCH				132
BMP6	ILKKYRNMVV	RA. CGCH				132
BMP7	ILKKYRNMVV	RA. CGCH				139

FIG. 1